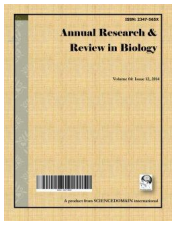

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Ontogeny of the *Lim* Code in the Mouse Gonads

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Authors' contributions

Author AD carried out sample collection, performed the experiments, analyzed the data, did statistical analysis and prepared the manuscript. Author DM conceptualized the project, analyzed the data and prepared the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Lim genes are developmentally regulated transcription factors involved in tissue specification and cell differentiation. Several *Lim* genes are present in the mammalian genome and each tissue expresses a unique pattern of *Lim* genes giving rise to the *Lim* code and it is believed that the *Lim* code is the determinant of the cell fate decisions taken by the tissue. In the developing mouse, *Lhx9* and *Lmo4* have been identified to play a key role in gonad specification and testis development; however the presence of other *Lim* genes and its co-regulators has not been reported. Recent evidences also suggest that the *Lim* genes are also expressed in other adult tissues. However, the presence of *Lim* genes in adult gonads has not been reported. In the present study we report the expression profiles of *Lhx9*, *Lmo 1-4* and *Clim 1* and *2* in the developing, neonatal and adult mouse gonads. Our results show that along with *Lhx9*, all the *Lmos* and *Clims* are expressed by the developing and adult gonads. With the exception of *Lmo4*, the expression of all these factors is sexually dimorphic with higher expression in the female gonads as compared to male gonads. Our results indicate that the *Lim* gene activity may be vital for ovarian development and functioning. It will be interesting to study the gonadal phenotypes of the *Lim* mutants to define their specific roles in the gonads.

Keywords: Lim genes; Lim Code; Lhx9; Clim; Lmo; gonads.

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1. INTRODUCTION

Gonad development in mammals involves a) Specification of the bipotential gonadal primordia b) its differentiation into the ovary or the testis and c) its structural organization. Functionally, the gonads during early development undergo rapid proliferation and differentiation until completely organized structurally and then enter a quiescence phase by birth. The gonads get reactivated at puberty under hormonal influences and function throughout the life to produce mature gametes. Several genes play a critical role in gonad specification and their differentiation into testis or the ovary [1]. Amongst these genes, are the homeobox class of transcription factors that are the key in cell specification and their differentiation [2]. While it was traditionally believed that these tissue organizer homeobox genes are expressed developmentally and they are silenced throughout life; recently this hypothesis has been revisited and it has been shown that many of these tissue specifying genes are reactivated in adulthood [3]. Intriguingly, a proper expression of these homeobox genes in adulthood is essential as their mis-expression is associated with tissue dysfunction and even cancer [4,5]. For example, in case of homeobox gene HOXA10 which is essential for uterine specification, it is expressed in the adult uterus and required for embryo implantation [6]. Loss of HOXA10 expression leads to infertility and endometriosis, while gain of its expression is observed in ovarian endometrioma [7,8]. Thus, it is essential to determine the ontogeny of the developmentally regulated genes beyond the stages of cell/tissue specification.

Amongst the various homobox genes involved in gonad development, the *Lim* homodomain gene *Lhx9* has been shown to be crucial for gonad specification. *Lhx9* is essential for development of the gonadal primordial as both male and female *Lhx9* knockout mice are infertile due to absence of gonads [9]. Developmental studies have shown that in the *Lhx9* -/- embryos, the gonadal primordial is developed until E9.5 but its development is arrested by E10.5 before the expression of *Sf1*. Indeed, corroborating the observations in the knockouts, in the mouse *Lhx9* mRNA is first detected in the gonadal primordia at E9.5 and its expression continues in the somatic cells until E12.5 [9]. Beyond the period of gonad specification, *Lhx9* mRNA continues to express in the gonads even after gonadal development with higher expression in the XY gonads as compared to XX gonads [10]. However, the expression of *Lhx9* beyond the period of sex determination and in adulthood has not been reported.

Lhx9 belongs to the *Lim* class of homeobox (*Lim-hd)* genes whose protein products contain two conserved N-terminal LIM domains fused to a homeodomain and it play important roles in cell fate decisions and brain development [11]. The activities of all LIM-HD proteins are controlled by LMO (LIM-only proteins) and CLIMS. LMO*s* are nuclear LIM-only proteins consisting of two LIM domains that are thought to act as molecular adapter molecules, linking proteins of various types together. The *Lim-only* genes occupy important biological roles in development and have also been shown to be involved in oncogenesis. For example, the LIM domains of the nuclear LIM-only protein LMO2 interact with DNA binding proteins like the members of the GATA, bHLH family [12] some of which are essential for gonadal development [13,14]. As the *Lmos* have only the *LIM* domain and not the DNA binding domain, they also control the activity of the *Lim-HD* genes by competing for its co-factors and interacting partners [15]. The co-factors of *Lim-hd* genes are CLIM molecules (co-factors of LIM*)* and are important for the activity of *LIM-HD* proteins [16]. *Clims* have been implicated in the synergistic activation or inhibition of *Lim-HD* target genes [16]. For example, in Drosophila, it has been demonstrated that the relative amount of Apterous and *Clim*/*Chip* expression is critical for proper wing development [17]. However, the mechanism by which the *CLIM* molecules exert their functions that lead to *LIM-HD* protein activity is not clear. Beyond regulation of *Lim-hd* functions, several lines of evidence suggest that the *CLIM* cofactor family have independent roles. Mutations in *Chip*, the Drosophila *CLIM* homologue, display severe segmentation disorders [15]; *Clims* are also known to have some roles in leukemia [18]. Thus, the *LIM* family of proteins together and independently plays key roles in many developmental processes. The entire Lim family and its co-regulators are evolutionary conserved and share extensive sequence and structural similarities and not surprising its regulation is also conserved across species [11].

In the context of gonadal specification, although *Lhx9* and other *Lim-HD* genes have been found to play an important role, little is known about the *Clims* and *Lmos* in gonadal development of

adulthood. Recently, *Lmo4* has been shown to be expressed in the mouse gonads in XY dominant manner during the window of sex determination and knocking down *Lmo4*, ex vivo affects *Sox9* expression [19]. These results imply that along with *Lhx9*, *Lmo* genes may also play a role in gonadal development. However, the expression profiles of other *Lmo*s and *Clim*s during the period of sex determination has not been studied. Also, the expression of *Lhx9*, *Clim*s and *Lmo*s post the window of sex determination and in adulthood has not been reported.

In this study, we report the ontogeny of *Lhx9*, *Clim1*, *Clim2* and *Lmo1-4* in developing and adult mouse gonads. We also compared its expression the levels of it expression at each stage in XX and XY gonads to gain an insight in to their regulatory roles in both the sexes across development. The results reveal that *Lhx9*, *Cims* and *Lmos* are not only expressed in the developing gonads, but also their levels are sexually dimorphic. Furthermore, our data also shows that these genes are robustly expressed in the adult gonads in a sexually dimorphic manner.

2. MATERIALS AND METHODS

2.1 Mouse Strains and Tissue Collection

The animals utilized for the following work has been approved by animal ethics committee of NIRRH (IAEC number: 08/08, 13/12). C57BL6 mice were housed in the experimental animal facility at NIRRH with 12 h day and night cycles at 25°C. Mature 8 week old female mice were housed 1:1 with males of same strain and the day of the vaginal plug was taken as E0.5. Pregnant mice were euthanized in the afternoon of E11.5, E12.5, E13.5, E14.5, E15.5 and E17.5; and the gonads were dissected. Gonads from Day 0 (at birth) pups and adult mice were also collected.

2.2 Embryo Sexing

Sex of the embryo was determined by PCR for *Jarid-1* [20]. The primer pairs designed for this region simultaneously amplify DNA fragments of 331 bp from the X chromosome homologue (*Jarid1c*) and 302 bp from Y chromosome (*Jarid1d*) thereby permitting resolution on standard agarose gels. The primer sequences along with annealing temperature and expected amplicon size are mentioned in Table 1.

Sexing of the embryos was done using PCR Terra PCR Kit (Clontech Laboratories, Inc., USA). A portion of the somatic tissue was solubilized in the solubilization buffer and incubated at 95°C for 10 mins, followed by neutralization. 5 µl of this extract was used to perform PCR reaction. PCR was performed on a standard thermal cycler (Veriti, Applied Biosystems) where initial denaturation was 95°C for 5 min, followed by 30 cycles of 95°C for 15 sec, 61°C for 15 sec, 72°C for 15 secs. The samples were run on 2% agarose gels and visualized under UV transilluminator. Samples that had two bands of 331 and 302 bp (corresponding to X and Y chromosome respectively) were designated as XY and those with only a single band of 331 bp were designated as XX (Supplementary Fig. 1).

2.3 Whole-Mount *In-Situ* Hybridization

2.3.1 Probe preparation

The plasmid for *Lhx9* was gifted by Dr. Shubha Tole (TIFR, Mumbai, India). It was linearized either using *Xho*1 or *Xba*1 (Thermo Fisher Scientific, USA) for antisense and sense probe respectively. Followed by purification, the probes were prepared and labeled with Digoxigenin (DIG) by *In-vitro* transcription with T3 polymerase for antisense probe and Sp6 for the sense probe using DIG RNA labelling kit (Roche Diagnostics, Germany). The ribo probes were purified and their labelling efficiency was analyzed using Dot-blot. The probes were used at a concentration of at 1ng/µl per reaction of *In-situ* hybridization.

2.3.2 *In-Situ* hybridization

Tissues were collected and fixed overnight in 4% paraformaldehyde (Sigma Aldrich, USA) in PBS at 4°C and dehydrated through a methanol gradient in PBST (0.1% Tween 20 in sterile 1X PBS). Samples were rehydrated through a methanol gradient and bleached using hydrogen peroxide (Sigma Aldrich, USA) in methanol followed by washings with PBST. The tissues were then digested in 10 mg/ml Proteinase K (RNase free) (Roche Diagnostics, Germany) solution in Tris EDTA buffer at 37°C for 10 min, followed by immediate fixation in 4% paraformaldehyde and 0.1% glutaraldehyde for 20 min at room temperature (RT). Samples were washed three times for 5 min each in PBST, rinsed in PBST-hybridization buffer (1:1), and washed once in hybridization buffer at RT. Samples were incubated in the hybridization

buffer consisting of 5X SSC, 50% formamide (Sigma Aldrich, USA) and 10% SDS (Sigma Aldrich, USA) for 2 h at 70°C. Digoxigenin labelled antisense or sense RNA probe (both at 1 ng/µl) was added to the solution, and samples were incubated in a water bath at 70°C overnight (12–16 h). Following day, the samples were washed with pre-warmed (70°C) hybridization buffer followed by 3 washes with PBST for 30 mins each at RT. Samples were then incubated at RT in blocking solution containing 20% heat-inactivated sheep serum (Sigma Aldrich, USA) in TBST buffer (0.1% Tween 20 in sterile 1X TBS and 0.05% Levamisol (Sigma Aldrich, USA)) followed by addition of alkaline phosphatase-conjugated anti-digoxigenin antibody (1:5000 dilution) and incubated at 4°C overnight. Next day, the samples were washed with TBST 5-7 times and left in TBST solution overnight. The tissues were finally washed with NTMT buffer containing Tris (pH 9.5), NaCl, $MgCl_2$, 0.1% Tween-20 and 0.05% Levamisol and incubated with alkaline phosphatase substrate (NBT/BCIP (Roche Diagnostics, Germany)) in NTMT buffer for color development (~5 h). The reaction was stopped at the appropriate color intensity by washing the samples in Tris EDTA buffer, followed by fixation in 10% formalin for 20 min at room temperature. The gonads were photographed under bright light on a stereomicrosope (Olympus, Japan)

2.4 RNA Isolation and Reverse Transcription

Gonads were homogenised in Trizol (Thermo Fisher Scientific, USA) and total RNA was extracted as per manufactures instructions. Following DNase1 (Roche Diagnostics, Germany) treatment for 30 min at 37C, one microgram of total RNA was reverse transcribed using MMLV reverse transcriptase enzyme and random hexamer primers using Advantage RT-PCR kit (Clontech Laboratories Inc., USA).

2.5 Quantitative Reverse Transcriptase PCR (qRT-PCR) Analysis

Primers that spanned exon-exon boundaries (except *18S rRNA* which does not contain introns) were designed using NCBI Primer-Blast software for all the mentioned genes. The sequences and the optimized annealing temperatures of the primers are mentioned in Table 1.

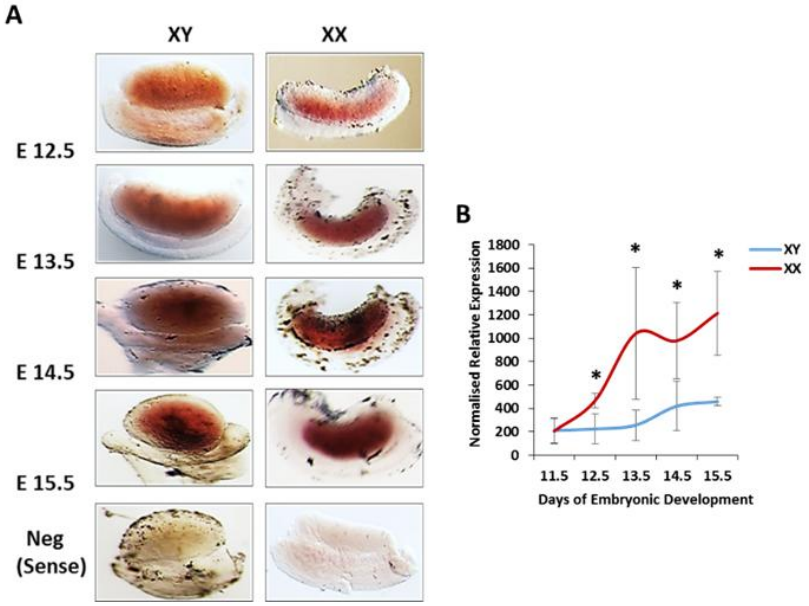

Fig. 1. *Lhx9* mRNA expression in the developing XX and XY gonads

*A: Relative expression of Lhx9 in the foetal XY and XX gonads at E11.5 - E15.5. The expression levels were normalized to 18S rRNA. Values are given as Mean ± SD. Student's T-test was used to show statistical significance, * indicates P< 0.05 when compared between XX and XY. B: Whole mount In-situ hybridization of XY and XX gonads at E12.5 to E15.5 using Lhx9 specific anti-sense and sense ribo probes. Magnification: 5X*

Table 1. Primer sequences with their respective annealing temperatures temperatures and expected size of PCR product

Gene	Accession number	Primer sequence	Exon No. of forward and reverse primer	Expected product size (bp)	Optimized annealing temperature (°C)
18S rRNA	NR_003278.3	F: 5'-AACCCGGTGAGCTCCCTCCC-3' R: 5'-TTCGAATGGGTCGTCGCCGC-3'	NA	114	68
Clim1	NM_010698.4	F: 5' AGAGGCCTTCACAAGAAGCA 3' R: 5' GACGGACAGCAAGTTCAACA 3'	Exon 8	115	64
Clim2	NM_001113408.1	For: 5' CAAGACCTACAGCCTCAGCC 3' Rev: 5' TGACATCTTCCGTTTCCTCC 3'	Exon 9 - Exon 10	130	64
Lmo1	NM_057173.3	For: 5' AATCAGAGATTTTGCGTGGG 3' Rev: 5' GATGGTGGGCGTTACTGAAC 3'	Exon 3 - Exon 4	125	63
Lmo2	NM_008505.4	For: 5' CTCAGCTGTGACCTCTGTGG 3' Rev: 5' ATCCTGACCAAAAAGCCTGA 3'	Exon 2 - Exon 3	111	63
Lmo3	NM_207222.2	For: 5' AGTGTGCCTGCTGCGACTGC 3' Rev: 5' AGGCAGCGCAGTTTCCCGTT 3'	Exon 3 - Exon 4	123	64
Lmo4	NM_010723.3	For: 5' CCAGAAGGTCTGCTGAGAGG 3' Rev: 5' ATGGTGCTGGCTACAAAGGT 3'	Exon 4 - Exon 5	120	64
Lhx9	NM_001025565.2	F: 5'-GGACCTCAAACAGCTTGCTC-3' R: 5'-AATTTTCAAACGTCGGGATG-3'	Exon 4 - Exon 5	103	60

qRT-PCR was performed using iQ SYBR green super mix (BioRad Laboratories Inc., USA) in triplicates on a BioRad CFX*9*6 Real-Time System (BioRad Laboratories Inc., USA). Briefly, the master mix containing the cDNA and appropriate primer pair, was subjected to initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, appropriate annealing for 30 sec and 72°C for 30 sec. The negative controls included wells without cDNA. All PCR amplifications were followed by melt curve analysis from 55°C to 95°C at the ramp rate of 3.3°C/sec with 0.5°C increments, 5 sec dwell time and a read at each temperature.

The homogeneity of the PCR amplicons was verified by electrophoresis on 2% agarose gels and also by analyzing the melt curves. *18S rRNA* amplification values were used for the normalization. The fold change in the expression of each gene was calculated using the formula $2^{\wedge-(\Delta Ct)}$ [21] using *18S rRNA* as the reference gene for normalization. Data reported are the mean fold changes ±SD for five biological replicates.

2.6 Statistical Analysis

Statistical significance between each samples was determined at $P < 0.05$ using an unpaired, two-tailed Student's T-test.

3. RESULTS

For all the genes, at the optimized annealing temperature, a single band of expected size (Table 1) were detected as evident by the agarose gel electrophoresis (Supplementary Fig. 2). The homogeneity of the products was further validated by melt curve analysis for all the genes. A single melt peak was detected in all the cases (Supplementary Fig. 3).

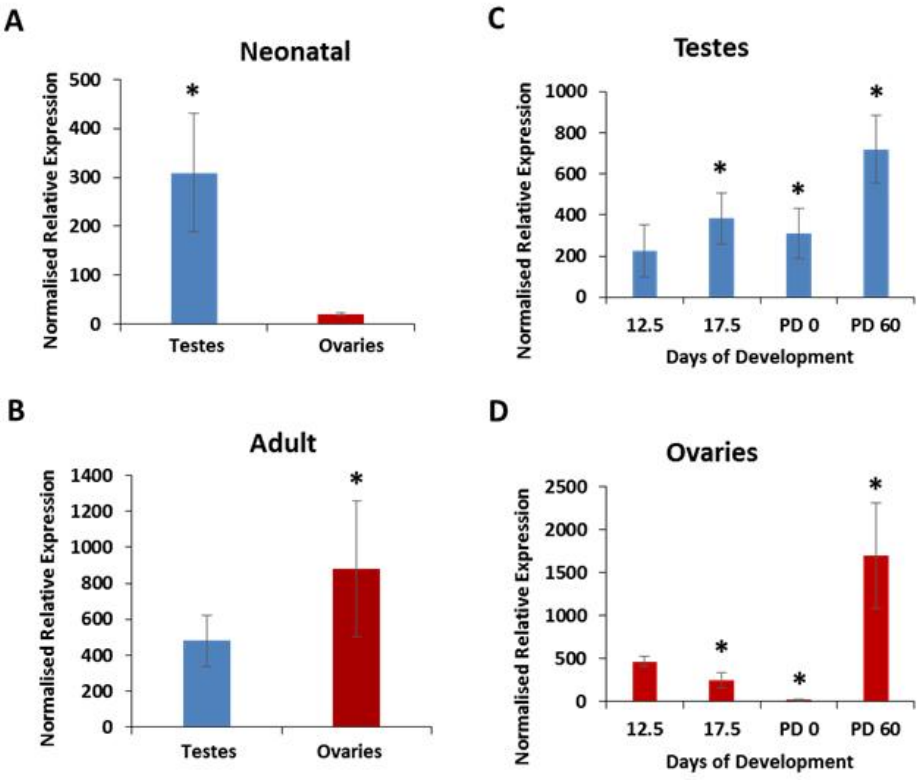

Fig. 2. Real time PCR based relative quantitation of *Lhx9* mRNA in the testes and ovaries from foetal life to adulthood

*A, B: Expression of Lhx9 in neonatal and adult gonads. C, D: Expression profiles of Lhx9 in the gonads from foetal stage to adulthood. In all the graphs, the expression levels were normalized to 18S rRNA. Y axis represents Mean ± SD of five biological replicates. * (A, B) indicates value is significantly different from the testicular sample (p< 0.05); * (C, D) indicates values statistically significant (P<0.05) as compared to E12.5*

3.1 Expression of *Lhx9* mRNA in the Developing Neonatal and Adult Gonads

As evident from Fig. 1A, *Lhx9* at E12.5 – E15.5 was expressed exclusively in the gonadal primordium and not in the supporting mesonephros when probed with an antisense *Lhx9* probe. Gonads incubated with sense probe for *Lhx9* did not show any expression. By whole mount *In-Situ* Hybridization, *Lhx9* was found to be expressed in a sexually dimorphic pattern, with higher levels in the XX gonads as compared to the XY gonads (Fig. 1A). By Real-Time PCR it was evident that *Lhx9* expression was identical in the XX and XY gonads at E11.5, after which the expression of *Lhx9* mRNA was significantly higher in the XX gonads showing a ~*2*.5 folds higher expression at E12.5. This XX dominant pattern of *Lhx9* expression continued with ~3-5 folds higher expression as compared to the respective expression levels in the XY gonads (Fig. 1B). All these differences were statistically significant (P<0.05).

In the neonatal gonads, *Lhx9* transcripts were significantly higher (P<0.05) in the testis as compared to ovaries. The expression of *Lhx9* mRNA in the testes was ~10 folds higher as compared to the ovaries (Fig. 2A). In the adult mice, the ovaries had a ~2 folds higher expression as compared to the testes (Fig. 2B) and these differences were statistically significant (p<0.05).

Comparing the expression of *Lhx9* in the testes from foetal to adulthood (Fig. 2C), it was evident that the expression of *Lhx9* is almost comparable between E12.5, E17.5 and at birth, whereas the levels increased by almost 2 folds in the adulthood. As compared to the values at E12.5 the difference in level of *Lhx9* was statistically significant (P<0.05).

In case of ovaries (Fig. 2D), as compared to E12.5, the *Lhx9* mRNA levels reduced steadily till birth; showing a 2 folds decrease at E17.5 and further a 10 folds decrease at birth. In the adult ovaries there was 3 folds increase in expression of *Lhx9* mRNA as compared to E12.5. These differences were statistically significant (P<0.05) When compared at all stages, *Lhx9* mRNA expression (in the testes as well as ovaries) was maximum in the adult testes and ovaries.

3.2 Expression of *Clim1* and *Clim2* mRNA in Foetal, Neonatal and Adult Gonads

Fig. 3A illustrates the expression profiles of *Clim1* and *Clim2* mRNA in the developing XX and XY gonads from E11.5 to E15.5. *Clim1* and *Clim2* were expressed in the XX dominant pattern during development. *Clim1* was sexually dimorphic from E11.5 to E15.5, showing ~2 to 3 folds higher expression in the XX gonads as compared to the XY gonads. These differences were statistically significant (P<0.05).

Clim2 was expressed at similar levels at E11.5 and became XX dominant after sex determination at E12.5. At E12.5, XX gonads had almost 2 folds higher expression as compared to the XY gonads. The significantly higher (P<0.05) levels of *Clim2* mRNA in the XX gonads were consistent and were ~*3* folds higher expression at E13.5, ~4 folds at E14.5 and almost 5 folds at E15.5, as compared to XY gonads at the respective days of embryonic development. At birth (Fig. 3B), the expression of *Clim1* and *Clim2* were ~10 folds higher in the XY gonads as compared to the XX gonads (P<0.05), whereas in the adulthood the levels of *Clim1* and *Clim2* mRNA in the XX and XY gonads remained identical (Fig. 3C).

3.3 Comparative Expression Pattern of *Clim1* and *Clim2* mRNA across Foetal, Neonatal and Adult Gonads

The expression levels of *Clim1* in the testes were comparable between E12.5, E17.5 and at birth, but the levels increased by ~8 folds at adulthood (Fig. 4). In case of ovaries, the expression of *Clim1* mRNA steadily decreased from E12.5 till birth, followed by a significantly higher (P<0.05) expression in the adulthood. As compared to at E12.5, there was ~2 folds decrease in expression of *Clim1* mRNA at E17.5, which further decreased by 5 folds at birth. In the adult ovaries, there was ~4 folds increase in the expression of *Clim1* mRNA as compared to at E12.5. These differences are statistically significant (P<0.05).

Levels of *Clim2* mRNA in the testes remained similar at E12.5 and E17.5 after which there was almost a 3 folds decrease at birth (P<0.05). In the adult testes there was a 3.5 folds increase (P<0.05) in the expression of *Clim2* mRNA as compared to at E12.5 (Fig. 4). In case of ovaries, like in testes, the level of *Clim2* mRNA remained

similar at E12.5 and E17.5 after which there was almost 40 folds reduction in its expression at birth and this reduction was statistically significant (P <0.05). In the adult ovaries, there was ~3 folds higher expression of *Clim2* mRNA as compared to ovaries at E12.5 (P <0.05). Both in the testes and ovaries, the expression of *Clim1* and *Clim2* mRNA were highest during adulthood (Fig. 4) and these differences were statistically significant (P <0.05).

3.4 Expression of *Lmo1*, *Lmo2*, *Lmo3* and *Lmo4* mRNA in Foetal, Neonatal and Adult Gonads

Fig. 5A shows that the expression levels of *Lmo1*, *Lmo2*, *Lmo3* and *Lmo4* mRNA in the developing gonads. The mRNA levels of all the four *Lmo* genes were similar in the XX and XY gonads at E11.5. At E12.5 and onwards, the expression of *Lmo1-3* was higher in the XX gonads, as compared to the XY gonads. Conversely, the expression of *Lmo4* mRNA was higher in the developing XY gonads from E11.5 to E15.5. The differences in expression of these genes between the two sexes are statistically significant (P <0.05).

In case of *Lmo1*, at E12.5 the expression was 4 folds higher in the XX gonads as compared to the XY gonads. The expression continued to be higher in the XX gonads showing ~3 folds increase at E13.5 and ~2 folds increase at E14.5 and E15.5 respectively as compared to XY gonads and these differences were statistically significant (P <0.05). *Lmo1* mRNA was maximally expressed at E14.5, after which the expression marginally dropped in the XX as well as XY gonads (Fig. 5A).

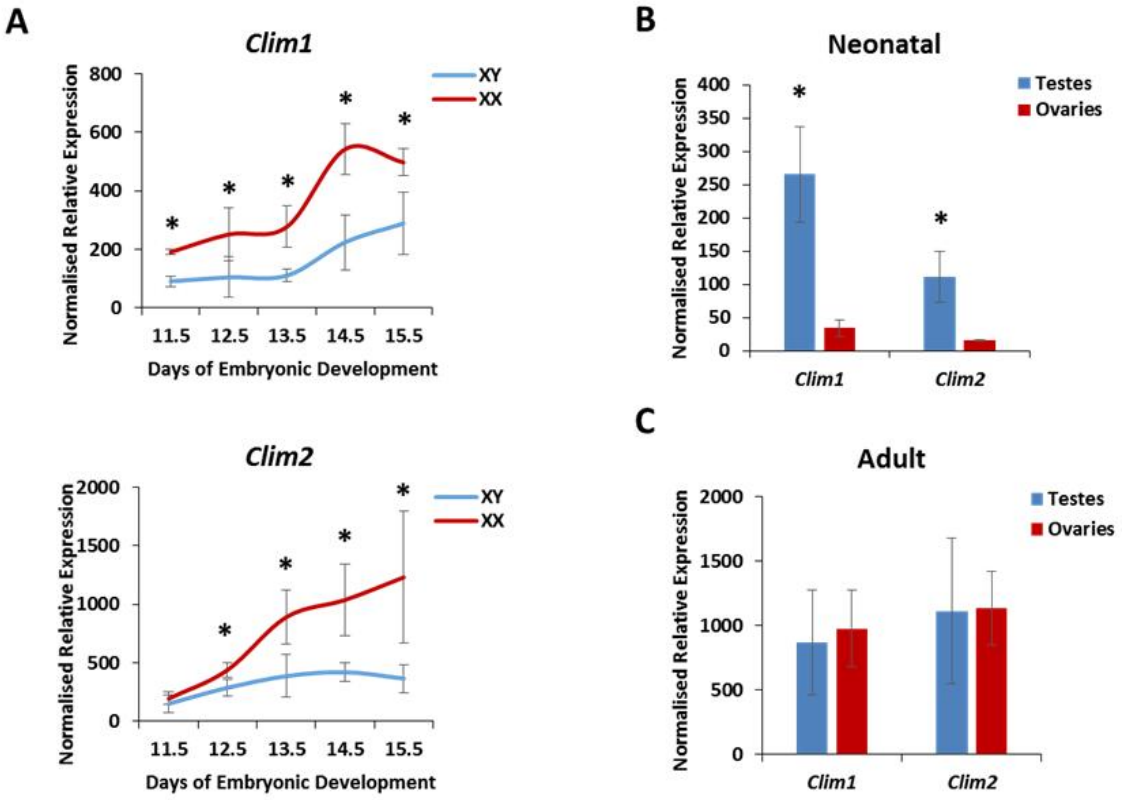

Fig. 3A, B, C. Relative expression of *Clim1* and *Clim2* across development from E11.5 to E15.5

*(A), in the neonatal (B) and adult (C) testes and ovaries. The expression levels were normalized to 18S rRNA. Values are given as Mean ± SD. Student's T-test was used to show statistical significance, * (A) indicates p< 0.05 when compared between XX and XY; * (B, C) indicates values statistically significant (P<0.05) when compared between testes and ovaries*

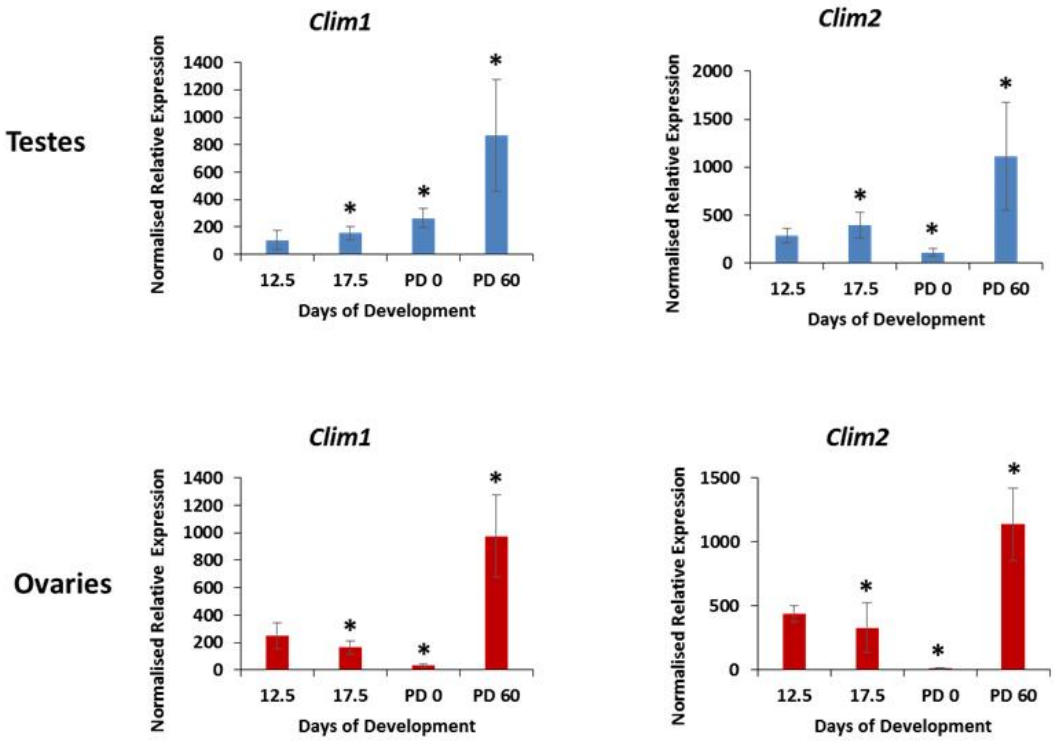

Fig. 4. Relative expression of *Clim1* and *Clim2* in the testes and ovaries at E12.5, E17.5, Day 0 and adulthood

*The expression levels were normalized to 18S rRNA. Values are given as Mean ± SD. * indicates values statistically significant (P<0.05) as compared to E12.5*

Lmo2 levels were detected to be almost 5 folds higher in the XX gonads as compared to the XY gonads at E12.5. This higher expression of *Lmo2* in the XX gonads continued across further days of development displaying a 3.5 folds increase at E13.5 whereas, a 2 folds increase at E14.5 and E15.5 respectively (Fig. 5A) as compared to XY gonads. These differences were statistically significant (*P* <0.05).

The expression of *Lmo3* in the developing XX gonads steadily increased from E11.5 to E15.5, whereas XY gonads showed baseline expression of *Lmo3* across development. In the XX gonads, at E12.5 and E13.5, there was almost 10 folds higher expression of *Lmo3*, which increased to almost 20 folds at E14.5 and E15.5 as compared to XY gonads at respective days of development (Fig. 5A). The differences at E12.5 – E15.5 between XX and XY gonads were statistically significant (*P* <0.05).

Lmo4 mRNA was expressed in the XY dominant pattern from E11.5 to E15.5. At E11.5, there was ~6 folds higher expression of *Lmo4* mR NA in the XY gonads as compared to the XX gonads. This elevated expression in the XY gonads was almost 2.5 folds higher at E12.5 and ~3.5 folds higher at E13.5, E14.5 and E15.5, as compared to the expression in the XX gonads at the respective embryonic days (Fig. 5A). These differences were statistically significant (*P* <0.05).

Fig. 5B gives the expression profiles of *Lmo1-4* in the newborn testes and ovaries. At birth, the expression of *Lmo1* was almost 2 folds and *Lmo2* was almost 4 folds higher as compared to the testes at birth. The differences in their levels were statistically significant (*P* <0.05). *Lmo3* was expressed at similar levels in the ovaries and testes in the newborns (*P* >0.05). In case of *Lmo4*, testes had 2 folds higher level of expression as compared to the ovaries. The differences were statistically significant (*P* <0.05). Quantitatively, amongst all the *Lmo* genes expressed at birth, the expression of *Lmo4* was highest followed by *Lmo2*, whereas *Lmo1* and *Lmo3* were expressed at similar levels (*P* >0.05).

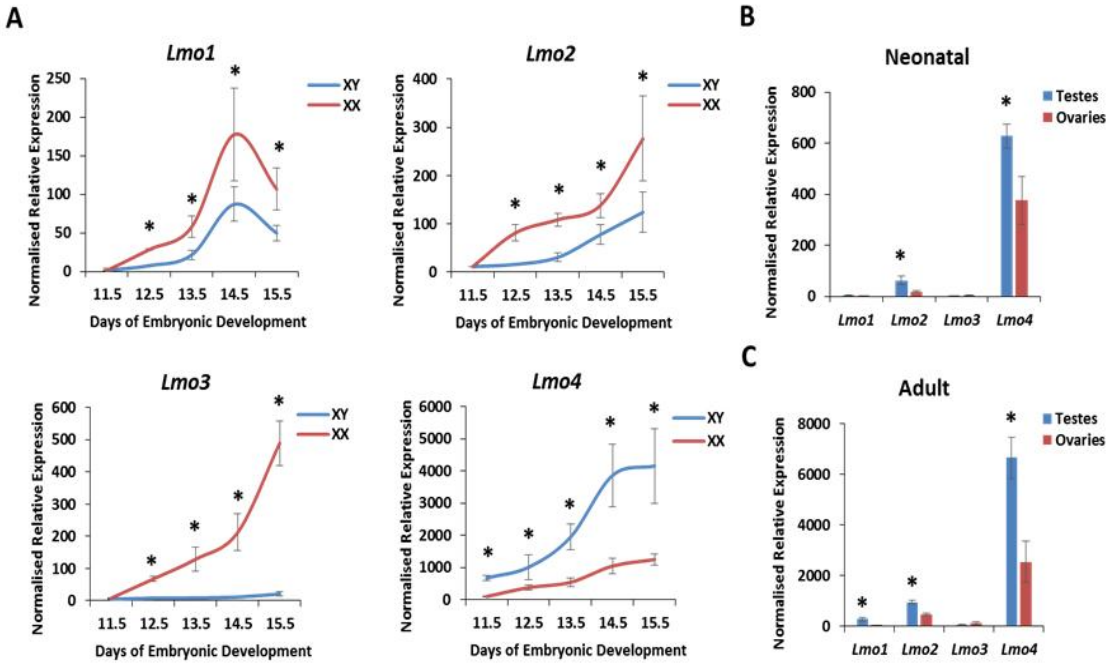

Fig. 5. Relative expression of *Lmo1, Lmo2, Lmo3* and *Lmo4* across development from E11.5 to E15.5 (A), in the neonatal (B) and adult (C) testes and ovaries

*The expression levels were normalized to 18S rRNA. Values are given as Mean ± SD. Student's T-test was used to show statistical significance. * (A) indicates P < 0.05 when compared between XX and XY; * (B, C) indicates values statistically significant (P <0.05) when compared between testes and ovaries*

In the adult gonads, *Lmo1*, *Lmo2* and *Lmo4* were dominantly expressed in the testes, whereas *Lmo3* was predominant in ovaries (Fig. 5C). In the testes, expression of *Lmo1* was 5 folds, *Lmo2* was 2 folds and *Lmo4* was 2.5 folds higher as compared to the ovaries. *Lmo3* mRNA was detected to be ~1.5 folds higher in the ovaries as compared to the testes. These differences were statistically significant (*P* <0.05).

3.5 Comparative Analysis of the Expression Profiles of *Lmo*s across Foetal, Neonatal and Adult Gonads

In case of testes, the levels of *Lmo1* mRNA remained similar at E12.5, E17.5 and at birth, whereas adult testes displayed ~30 folds higher expression. The differences were statistically significant (*P* <0.05). In ovaries, the expression of *Lmo1* gradually decreased as the development proceeds from E12.5 till birth. As compared to E12.5, at E17.5 there was a 4 folds reduction in the expression of *Lmo1* which further reduced by ~3 folds at birth. Adult ovaries showed highest expression of *Lmo1* mRNA which was ~2 folds higher as compared to E12.5 (Fig. 6). The difference in the level of expression in adults as compared to E12.5 were statistically significant (*P* <0.05).

In the testes, as compared to E12.5, *Lmo2* expression increased by 10 folds at E17.5 (*P* <0.05) and the level of expression remained similar at birth (*P* >0.05). In the adult testes, there was almost 100 folds higher expression of *Lmo2* as compared to at E12.5 and was statistically significant (*P* <0.05). In case of ovaries, as compared to at E12.5, the expression of *Lmo2* was almost 3.5 folds higher at E13.5. Minimal level of *Lmo2* mRNA was detected in the ovaries at birth, but the expression escalated almost 40 folds in the adult ovaries (Fig. 6). These differences were statistically significant (*P* <0.05).

Expression of *Lmo3* in the developing testes at E12.5 and E17.5 was identical, but the expression reduced almost 3 folds at birth and increased by ~7 folds in the adult testes as compared to the developing testes at E12.5. These differences were statistically significant (*P* <0.05). In case of ovaries, as compared to E12.5, *Lmo3* expression was ~3 folds higher at E17.5, but the expression was minimal at birth.

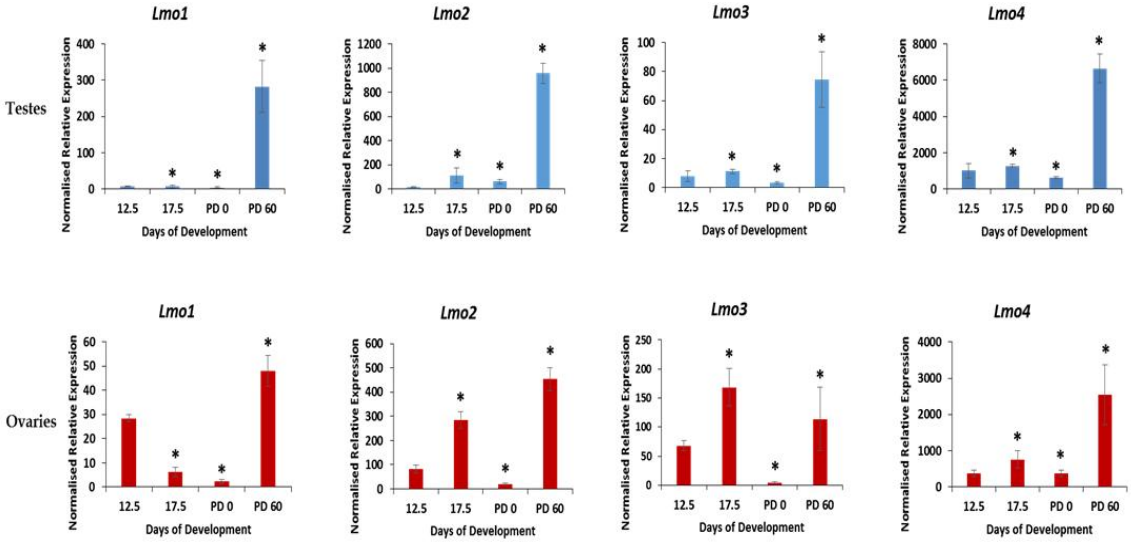

Fig. 6. Relative expression of *Lmo1, Lmo2, Lmo3* and *Lmo4* in the testes and ovaries at E12.5, E 17.5, Day 0 and Adulthood

*The expression levels were normalized to 18S rRNA. Values are given as Mean ± SD. * indicates values statistically significant (P <0.05) as compared to E12.5*

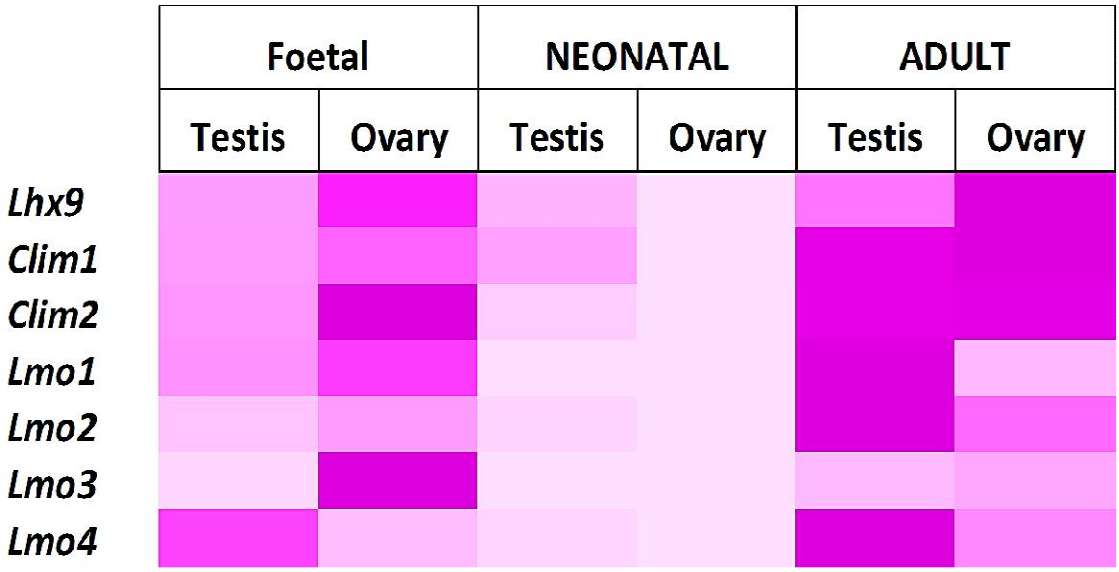

Fig. 7. The *Lim* code of the mouse gonads

The heat map was generated based on the levels of expression of each gene labeled on left in the testis and ovary at foetal (E12.5), neonatal and adult stages. Each shade represents the intensity value. The intensity of color light to dark represents low to high expression

In the adult ovaries, the expression of *Lmo3* again escalated and was almost 2 folds higher as compared to at E12.5 (Fig. 6). These differences were statistically significant (*P* <0.05).

The expression levels of *Lmo4* mRNA in developing testes at E12.5 and E17.5 were similar and the expression decreased by almost 2 folds at birth. In the adult testes, the expression of *Lmo4* was maximum, which was ~6 folds higher as compared to at E12.5. These differences were statistically significant (*P* <0.05). In case of ovaries, the expression of *Lmo4* mRNA was almost 2 folds higher at E17.5

as compared to E12.5 (P <0.05). At birth, the expression of *Lmo4* mRNA was reduced by almost 2 folds and the expression was equivalent to that at E12.5 (P <0.05 E12.5 vs PD 0). Maximum *Lmo4* mRNA was detected in the adult ovaries, which was almost 7 folds higher as compared to at E12.5 (Fig. 6). These differences were statistically significant (P <0.05).

3.6 *Lim* Code for the Gonads

Fig. 7 illustrates the expression pattern of *Lhx9* and its co-regulators *Clim (1-2)* and *Lmo (1-4)* in the developing (E12.5), neonatal and adult gonads. The plotted heat-map represents the dynamic pattern of expression for these genes depending upon their Real-Time PCR based expression values. The intensity of the color depicts the level of gene expression in the testes and ovaries at a particular stage of development, ie, darker the intensity corresponds to higher the expression of a respective gene at a specific time point. It is evident from the map that during the development, all genes other than *Lmo4* were predominantly expressed in the XX gonads; *Lmo4* was XY dominant. Interestingly, expression of all the genes dipped during the neonatal stage and escalated again during the adulthood, which showed maximum expression, with an exception of *Lmo3* which was maximally expressed during the developmental stage. In general during development, the *Lim* genes seem to be highly expressed in the developing ovaries as compared to testes, the cluster seems to be quiescent at birth and reactivated in adulthood. In the adult gonads, the *Lim* genes are active in both testes and ovaries, with *Lhx9* being ovary dominant and *Lmo 1, 2* and *4* being testes dominant. *Clims* are equally expressed in both the gonads.

4. DISCUSSION

LIM homeobox genes and their co-regualtors are evolutionary conserved and share extensive sequential, structural and regulatory similarities [11]. *LIM* homeobox genes belong to a subfamily of homeobox genes which encode LIM-homeodomain (LIM-HD) proteins. LIM-HD proteins have two LIM domains in their amino termini and a centrally located HD that is used to interact with specific DNA elements in target genes. The LIM domain contains two tandemly-repeated, cysteine-rich, double-zinc finger motifs that can be recognized by a number of co-factors which can mediate LIM-HD functions thus, they participate in a wide array of developmental events. LMO (LIM-only) are closely related to the LIM-HD (LIM-homeodomain) proteins from a family of proteins that is required for myriad developmental processes and also contribute to diseases such as T-cell leukemia and breast cancer [22]. The four LMO and 12 LIM-HD proteins in mammals are expressed in a combinatorial manner in many cell types, forming a transcriptional '*LIM* code'. These activities of *Lim-HD* genes are controlled by their co factors *Clims* and at the functional level, *Lmo* (*LIM* only) and *Clim* (co-factor of LIM*)* proteins regulate the action of *LIM-HD* molecules. To determine the combinations in which members of the mouse *Lim-HD*, *Lmo* and *Clim* families may function in the gonads, a comparison of the expression of these genes is essential. Furthermore, to suggest possible roles for these genes in the gonads, their expression patterns must be analyzed in the context of the stages of maturity of gonadal cells at different time points.

In this study we have addressed the expression profiles of *Lhx9*, the two *Clim* and the four *Lmo* genes in the developing and postnatal mouse gonads. We have also studied its expression in the XX and XY gonads across development to gain an insight in to their regulatory functions in ontogeny of the reproductive system. We studied their expression by real time RT-PCR where the expression was normalized using *18S rRNA* as housekeeping gene.

Choice of housekeeping genes is highly controversial and several investigators have used different genes for normalization. Previous studies in our laboratory have shown that as compared to *Gapdh* and *Sdha*, *18S rRNA* expression is robust and show minimal variability in the gonads of both the sexes (Supplementary Fig. 4). In the context of developing gonads we and others have shown that *18S rRNA* is the most appropriate gene for normalization [23,24]. Therefore in this study we chose to use *18s rRNA* levels for normalization and determine the changes in expression of *Lim* genes during development and adulthood.

4.1 *Lhx9*

Lhx9 is one of the members of the *LIM-HD* family that is expressed in multiple tissues and has widespread functions. However, mice knockout for *Lhx9* are viable suggesting that the gene is dispensable in most tissues. However, the *Lhx9* -/- mice are infertile and the infertility is due to failure of gonad development [9] suggesting its

central role in gonadal development. Birk et al. [9] had previously reported that *Lhx9* is expressed in the genital primordium at E10.5 and is detected until E12.5. Extending these observations further and corroborating the data reported by Bouma et al. [10], we herein observed that *Lhx9* is expressed in the developing mouse gonads during entire window of sex determination (E11.5 - E15.5). We further report that *Lhx9* expression is sexually dimorphic with higher expression in the XX gonads as compared to XY gonads as reported earlier [24]. Irrespective of the gestational age, *Lhx9* was higher in the XX gonads as compared to XY gonads. These observations are in contrast to those reported by Bouma, et al. [10] where the expression of *Lhx9* was reported to be XY dominant. At present, we do not have an obvious explanation of such contrasting finding; differences in housekeeping genes used for normalization, presence of some splice variants or strains of mice used could be a possibility. To verify if the expression of *Lhx9* is indeed sexually dimorphic with higher expression in XX vs XY gonads, we performed whole mount *in-situ* hybridization for *Lhx9* transcripts form E12.5 - E15.5 in the XX and XY gonads. In all these cases, *Lhx9* was exclusively detected in the gonad primordium and not the mesonephros and the signals were higher in the XX gonads as compared to XY gonads. Thus we conclude that *Lhx9* is expressed higher in the developing ovaries as compared to testes and may have some regulatory functions in ovarian development.

We next tested the expression of *Lhx9* in the postnatal and adult testes and ovaries to determine if it had any roles beyond the period of sex determination and differentiation. Our results revealed that *Lhx9* transcripts are detected in the postnatal testes and ovaries. However on the day of birth, *Lhx9* mRNA was higher in the testes as compared to ovaries, however in the adults the expression of *Lhx9* was higher in the ovaries as compared to testes. Comparing the developmental profiles, it was evident that *Lhx9* was robustly expressed in the developing ovaries mainly at E12.5 which coincides with the timing of ovarian specification. The expression of *Lhx9* was downregulated once the ovary is formed and remained quiescent until reactivated in adulthood. Such downregulation of *Lhx9* in the pre-pubertal mouse brain has also been reported [11]. These results suggest that the expression of *Lhx9* persist beyond the period of development and may have some functions in the adulthood. The sexually dimorphic pattern of expression further suggests that the *Lhx9* regulated transcriptome may have specific roles in the adult ovaries. While the roles of *Lhx8* in the developing and adult mouse ovaries have been reported earlier [25,26], this is the first study reporting the presence of *Lhx9* in the adult ovary. Since mice null for *Lhx9* do not develop gonads, its functions in adulthood are difficult to determine. Conditional gonad specific knockout mice would be required to evaluate time and cell type specific functions of *Lhx9* in the adults.

4.2 *Clims*

The *LIM* domain is a tandem zinc finger motif that serves as an interface for protein-protein interactions; found in a variety of protein families, including the *LIM*-homeodomain (*Lhx*) and *Lim*-only (*Lmo*) transcription factors [25,27]. Initially identified as a co-activator of *Lhx* and *LMO* [16,28], *Clims* co-ordinate the organization of transcriptional complexes through the amino-terminal homodimerization domain and the carboxy-terminal *LIM*-interacting domain (LID) [16,28]. Additionally, *Clims* can associate with other DNA-binding proteins, including *GATA, bHLH* and *Otx* family members [28]. The *Clim* family consists of two highly conserved members: the ubiquitously expressed *Clim2*, and the more regionally expressed *Clim1* [16]. Germline deletion of *Clim2* in the mouse causes embryonic lethality by day E9.5 [29], while *Clim1* knockout mice display no developmental defects (http://www.informatics.jax.org/). In general, *Clims* are thought to play a key role in brain [30,11], hair follicles [31] and breast [31] and is associated with breast and other cancer [18,32]. The presence of *Clims* in the gonads has not been investigated. In this study, for the first time we report that both *Clim1* and *Clim2* are expressed in the developing and adult mouse gonads. Like *Lhx9*, both *Clim1* and *Clim2* are expressed in the sexually dimorphic manner with higher expression in XX as compared to XY gonads during the window of sex determination and gonad differentiation in the mouse. Unlike *Clim1* which is sexually dimorphic at E11.5, *Clim2* becomes sexually dimorphic after E12.5. Nevertheless, by the time the ovary is specified (E12.5), both *Clim1* and *Clim2* are higher in the XX gonads as compared to XY gonads.

Similar to that of *Lhx9*, in the neonates, both *Clim1* and *Clim2* mRNA were detected; the expression was higher in the testis as compared to ovary. However, unlike *Lhx9*, in adulthood the

expression of *Clim1* and *Clim2* was similar in both testes and ovaries. Comparing the developmental profiles of *Clims*, it was evident that both the *Clims* are expressed in the developmental stages and its expression is highest in adulthood. Of the two *Clim* factors, the expression of *Clim2* mirrored to that of *Lhx9* with robust expression during the stages of gonadal development, and then reactivation in adulthood. Since *Lhx9* is reported to strongly interact with both *Clim1* and *Clim2* [33], it is likely that there might be competition between the two *Clims* with *Lhx9* during the entire period of development and regulate differential functions.

4.3 *Lmos*

LMO proteins are nuclear transcription co-regulators characterized by the exclusive presence of two tandem *LIM* domains and no other functional domains. Four *Lmo* genes have been identified; that regulate gene transcription by functioning as scaffolding proteins by mediating protein-protein interactions. Together, these proteins play important roles in cell fate determination, cell growth and differentiation, tissues patterning and organ development. The *Lmos* also interact with *LIM-HD* proteins and form multimeric complexes and different combinations of *LIM-HD*, *LMO* and co-factor proteins are important in specifying a wide range of different cell types [28]. In recent years, the *Lmos* are also shown to be involved in cancer, fibrosis [32] and also epithelial to mesenchymal transition [34] suggesting their widespread roles beyond development.

In the context of gonadal development, the expression of *Lmo4* has been reported. *Lmo4* mRNA has been found to have higher expression in the XY gonads as compared to XX gonads by E11.5 [19]. Lentiviral mediated downregulation of *Lmo4* in cultured mouse gonadal cells led to loss of expression of genes *Sox9* and *Amh*, suggesting that *Lmo4* may have key roles in Sertoli cell specification. Corroborating the data reported by Munger, et al. [19], we also observed higher expression of *Lmo4* in the XY gonadal primordia as compared to XX from E11.5 until E15.5. In contrast to *Lmo2* however *Lmo1*, *2* and *3* were found to be expressed in an XX dominating manner. All the three *Lmos* were found to be higher in the XX gonadal primordia from E12.5 and remained sexually dimorphic until E15.5. This suggests that the *Lmo1-3* might have an opposing role as compared to *Lmo4* which has a role in Sertoli cell development.

Beyond foetal development, mRNA for all the *Lmos* were detected in the neonatal and adult gonads. Interestingly, *Lmo4* was always expressed higher in the testes as compared to the ovaries suggesting its conserved roles during different stages of development. Unlike *Lmo4*, *Lmo1* and *2* that were expressed in the XX dominant manner in the foetal gonads, were XY dominant in adulthood. *Lmo3* however was present in almost equal amounts in both XX and XY gonads.

Like *Lhx9* and *Clims*, the expression of *Lmos* also changed dynamically across development. All the four *Lmos* were robustly expressed in the adult testes as compared to all other stages; the ovaries however had more dynamic changes. *Lmo1* was robustly expressed at the time of sex determination and its expression reduced thereafter and reached nadir at birth, and then reactivated in adulthood. *Lmo2* expression increased after sex determination, reduced at birth and increased thereafter. *Lmo3* expression was least dynamic and the expression remained with an order of magnitude irrespective of stage of development except at birth where the expression was lowest. *Lmo4* expression in the ovaries remained constant through development and increased maximally at adulthood. These observations point towards dynamic roles of these genes in gonadal development and adult functions like that in the brain.

4.4 The *Lim* Code of the Gonads

According to the "*Lim* code" hypothesis, a particular combination of *Lim* genes is expressed that specify distinct cell fate. In mammals, different combinations of the *Lim* genes are expressed in a combinatorial fashion during development in many tissue types forming a unique *Lim* code. While the *Lim* code of the developing brain and nervous system has been reported, the *Lim* code of the gonad does not exist. In this study we devised the *Lim* code of the gonads using a heat map based on the level of the expression of the genes in the XX and XY gonads at each stage of development. As evident, most *Lim* genes (except for *Lmo4*) were highly expressed in the developing ovary as compared to the testis. In the neonatal period the *Lim* genes undergo quiescence only to be reactivated in adulthood. In the adult gonads, *Lhx9*, *Clim1* and *Clim2* are most abundant in the ovaries whereas the *Lmo* genes are more abundant in the testes. Intriguingly, *Lmo3* was most abundant in the foetal ovaries as compared

to rest of the stages. These results together suggest that the *Lim* genes (specifically *Lmo3*) may be associated with ovarian development; *Lmo4* is involved in testicular development. Once the gonad is specified the *Lim* genes undergo quiescence and then get activated in adulthood in a gender specific manner. In the adults, the *Clims* and *Lmos* may be dominantly involved in testicular functions, *Lhx9* and *Clims* may be involved in ovarian functions. To the best of our knowledge, although not complete, this is the first study reporting *Lim* code of the mammalian gonads.

5. CONCLUSION

In conclusion, our results for the first time have shown that the entire complement of the *Lim* co-regulators are expressed in the gonads and their expression is sexually dimorphic. The *Lim* gene activity seems to be required for ovarian development and also involved in adult ovarian functioning. It will be of interest to look at the gonadal phenotypes of the *Lim* mutants to define their specific roles in gonadogenesis during development and gametogenesis in adulthood.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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